Molecular phylogenetic analysis of Pederson's cleaner shrimp *(Ancylomenes pedersoni)* supports classification of Bermudan population as separate species (*Ancylomenes anthophilus)*

Honors Research Thesis

Presented in Partial Fulfillment of the Requirements for graduation "with Honors Research Distinction in Evolution and Ecology" in the undergraduate colleges of The Ohio State University

By
Spencer Palombit

The Ohio State University
April 2014

Project Advisors:
Dr. Marymegan Daly, Department of Ecology, Evolution, and Organismal Biology,
Benjamin Titus (PhD Student), Department of Ecology, Evolution, and Organismal Biology

Abstract

Caribbean populations of the Pederson's cleaner shrimp (*Ancylomenes pedersoni*) have undergone increasing phylogenetic scrutiny, especially in Bermuda and the Florida Keys. Until now, however, no research using molecular data has been conducted to evaluate whether these populations constitute separate species. In this study, phylogenetic analyses of Pederson shrimp from the Florida Keys and Bermuda were performed using multiple loci: mitochondrial genes encoding cytochrome c oxidase subunit I (COI) and 16S-rDNA, and nuclear genes encoding the enzyme enolase. The phylogenetic networks estimated using mitochondrial DNA strongly suggest that the populations constitute three reciprocally monophyletic groups, one endemic to Bermuda, and two sympatric clades in the Florida Keys. The insights gained from this study will be instrumental in resolving the contested status of the Pederson's cleaner shrimp species, and will contribute to a fuller understanding of Caribbean species diversity.

Introduction

Species delimitation is one of the prime goals of phylogenetics, but the issue of what constitutes a species has been a problematic and disputed question (Mayden 1997; de Queiroz 1998). Many definitions of the proper classification of a species have persisted; phylogenetic, biological, character-based, and numerous other species concepts are considered competing and valid possibilities (de Queiroz 2007; Hey 2006). Even within a particular species concept, different methods and criteria for species delimitation may be chosen. In the phylogenetic approach, the species concept adhered to in this study, either morphological or molecular genetic data may be used to delimit species (Larson 1998; Shaffer et al. 1997). The phylogenetic identification of cryptic species, defined as populations that show significant genetic divergence while displaying little or no morphological differences, can thus present a formidable challenge. With the advent of advanced molecular assays, though, cryptic species are being increasingly realized in many ecosystems and represent a growing field of phylogenetic inquiry (Held 2003; Machordom & Macpherson 2004; Plaisance et al. 2009). Furthermore, in an era that many conservationists have termed the "Sixth Extinction" (Leakey & Lewin 1996; Pimm & Brooks 2000), identifying cryptic or endemic species is crucial for producing viable conservation plans tailored to the specific organism at risk (Perez-Losada et al. 2002; González et al. 1998).

In the Caribbean, cryptic species diversity is of particular import given the nature of island biogeography, as isolated islands may foster multitudes of endemic species. The species delimitation of Caribbean marine invertebrates has often proved a challenging task, though, as the marine environment actually allows broad larval dispersal strategies that

promote panmixia, but host specificity requirements and strong currents create reproductive restriction and isolation (Tolley et al. 2005; McMillan-Jackson & Bert 2004; Palumbi 1994). Additionally, the study of such insular ecosystems may allow crucial insight into how endemism evolves and how endemic species will react to the threat of climate change, ocean acidification, and invasive species (Briggs 1966; Jansson 2003; Sodhi et al. 2004).

Although Bermuda is relatively geographically isolated in the North Atlantic, (≈1800 kilometers from the Florida Keys and ≈1400 km from the northernmost Caribbean reef systems) it lies in the path of the Gulf Stream and so maintains connections to Florida and the Caribbean through this water mass. This current steadily transports warm water and some marine biota from the Caribbean to Bermuda (Iliffe et al. 1983). This regular flow of organisms (both larvae and adults) from the wider Caribbean to Bermuda may diminish the potential for endemism in Bermuda, as gene flow from other marine populations is easily facilitated by the current (Schultz & Cowen 1994; Hare et al. 2002; Sterrer & Schoepfer-Sterrer 1986). Conversely, though, there has been a growing recognition that Bermuda may possess more endemic crustacean species than previously thought (Iliffe et al. 1983; Bowman & Iliffe 1985; Fosshagen & Iliffe 1985; Boxshall & Iliffe 1986).

Pederson's cleaner shrimp, *Ancylomenes pedersoni*, are common throughout the Caribbean and Western Atlantic reef systems (Chace 1958) and are found extensively in Bermuda. They are primarily symbiotically associated with the corkscrew anemone (*Bartholomea annulata*), but also can readily be found on the giant Caribbean anemone (*Condylactis gigantea*) and the branching anemone (*Lebrunia danae)* (Briones-Fourzan et

al. 2012). Client fish cleaned by these shrimp include groupers, parrotfishes, tangs, and goatfishes (Wicksten 1995a), which they attend to at their anemone cleaning stations (Silbiger & Childress 2008). Although the details of its reproductive biology are unknown, as a tropical caridean shrimp, *A. pedersoni* is expected to reproduce continually throughout the year and releases planktotrophic larvae (Bauer 2004).

The identity and distinctiveness of the Bermudan population of Pederson's cleaner shrimp has been under examination for some time. Holthuis & Eibl-Eibesfeldt (1964) offered an initial designation of the population as a new species, *Periclimines anthophilus,* based on the anteriority of the hepatic spine and the form of the second pereiopod, which differ in *Periclimines pedersoni* (now *A. pedersoni).* Spotte (1999), however, posited that the Bermudan population's morphological differences are too indistinct to justify separation, and Wicksten (1995b) concluded that it was no more than a junior synonym of the Pederson's cleaner shrimp lineage on similar grounds. More recently, Okuno and Bruce (2010) revived the argument that the minute morphological differences that exist between the Bermuda population and its Caribbean neighbors are significant enough to warrant its designation as a separate species, now named *Ancylomenes anthophilus*. This study uses genetic data to resolve phylogenetic questions about the distinctiveness of *Ancylomenes anthophilus* with respect to *A. pedersoni,* by comparing sequences from two mitochondrial genes and one nuclear gene amplified from Bermudan and Floridian populations.

Materials and Methods

Sample Collection:

Bermudan samples of *A. pedersoni* (n=35) were collected via SCUBA from 6 coral reef locations, at depths between 4 and 15 m (see Fig. 2). The samples were primarily harvested from the anemone host *Condylactis gigantea,* but three samples each were also collected from *Bartholomea annulata* and *Lebrunia danae*. The shrimp were gathered by hand, and placed in individual whirl-bags for transport back to the Bermuda Institute of Ocean Science (BIOS), where they were preserved in RNA later. These samples were then delivered to the Ohio State University for DNA analysis and PCR.

All samples from Florida were originally collected by Titus & Daly (2015) (see Fig. 2). These shrimp (n=94) were collected via SCUBA from 10 coral reef sites, at depths between 9 and 18 m. The sites covered four distinct geographic regions in Southeast Florida: Fort Lauderdale (FT), Upper Keys (UK), Middle Keys (MK), and Lower Keys (LK). All of the samples were harvested from the anemone host *B. annulata*. These samples fall into at least two distinct lineages (Clades 1 and 2); only clade 1 includes populations outside of Florida at present (Titus & Daly 2015). All samples were preserved in 100% EtOH in the field and transferred to The Ohio State University for DNA extraction and PCR.

DNA Extraction, PCR, and Sequencing:

The study focused on three loci: mitochondrial genes encoding cytochrome c oxidase subunit I (COI) and 16S-rRNA (16S), and nuclear genes encoding the enzyme enolase. COI and 16S data for the Floridian samples were published by Titus & Daly (2015). For both those samples and the newly collected Bermudan shrimp, total genomic

DNA was isolated using DNeasy Blood and Tissue Kits (QIAGEN Inc.) and stored at -20 °C. Using the universal primers LCO1490 and HCO2198, a ~650-bp-long fragment of the mtDNA cytochrome c oxidase subunit I (COI) gene was amplified using PCR. Reactions were carried out in 25 µl volumes using IllustraTMpuReTaqTM Ready-To-GoTM PCR beads (GE Healthcare) with final concentrations of 25–40 ng template DNA, 200 µM of each deoxyribonucleotide triphosphate (i.e. dATP, dCTP, dGTP, dTTP), 10 µM Tris–HCL, 50 µM KCL, 1.5 µM MgCl2, 2.5 U puReTaq DNA polymerase and reaction buffer, and 0.2 µM each of primers LCO1490 and HCO2198. PCRs were performed in an epigradient Mastercycler (Eppendorf) with run conditions following Santos (2006). 16S-rDNA was amplified using the primers 16SA-Shrimp (5′-ACTTGATATATAATTAAAGGGCG-3′) and 16SB-Shrimp (5′-′CTGGCGCCGGTCTGAACTCAAATC-3′). PCR was performed at 95 °C for 5 min, then 30 cycles of 92 °C for 1 min, 55 °C for 45 s and 72 °C for 1 min, and a final extension of 72 °C for 1 min.

Sequences for enolase were amplified from new and existing DNA samples using the primers EA2/ES2 (from Tsang et al. 2011), and run conditions from DeGrave et al. (2014). The thermal cycle had an initial denaturation step for 3 min at 94 °C, followed by 33 cycles of 30 s at 94 °C, 30 s at 53 °C and 50 s at 72 °C, with a final extension at 72 °C for 3 min (DeGrave et al. 2014).

Samples from all loci were cycle sequenced in both directions at Beckman Coulter Genomics (Danvers, MA, USA). Consensus sequences were created for each sample for each locus using SEQUENCHER 4.9 (Gene Codes Corp., Ann Arbor, MI, USA). Consensus sequences were aligned using MUSCLE (Edgar 2004) in MEGA 5.2.2 (Tamura et al. 2011). Low-confidence sequence reads were trimmed from each end of the aligned

consensus sequences to produce final sequences. The final sequence length for COI was 590 base pairs, the final sequence length for 16S was 389 base pairs, and the final sequence length for enolase was 350 base pairs. Final sequences for COI and enolase were translated into amino acids to confirm that no stop codons were present within the open reading frame. Each sequence was then verified as a crustacean source using a nucleotide BLAST query against the nr database in GenBank.

Phylogenetic Analysis and Species Delimitation:

The aligned sequences were run through a suite of phylogenetic analyses to illuminate the relationships between the populations, as congruence across a plurality of phylogenetic approaches is desirable (Carstens et al. 2013; Pante et al. 2015). Per site mutations rates for each locus were taken from the extant literature on crustacean genomics: 1.7×10^{-8} for COI (Lessios 2008), 8.5×10^{-9} for 16S (Williams & Knowlton 2001), and 1.0×10^{-9} for enolase (Moriyama & Gojobori 1992; Kumar & Subramanian 2002; Hurt et al. 2009). A per site mutation rate of 1.7×10^{-7} was used for the multilocus concatenated data set, taken from the calculation by Hurt et al. (2009) of the geometric mean across multiple loci for alpheid shrimp.

The sequence data for each locus was run through DNA/Protein model analysis in Mega 5.2.2 to find the mutational model with the greatest likelihood. The best maximum likelihood phylogenetic tree was determined for each locus by applying the mutational model in the maximum likelihood evaluation in MEGA 5.2.2. Branch supports were estimated by 1000 bootstrap replicates.

The single locus data were also analyzed using Bayesian Evolutionary Analysis by Sampling Trees (BEAST: Drummond & Rambaut 2007; Suchard & Rambaut 2009), run

on XSEDE through the CIPRES Science Gateway supercomputer portal (https://www.phylo.org/), to produce rooted, time-measured Bayesian phylogenetic trees (referred to as "Bayesian phylogenetic trees" for the rest of the paper). MCMC analyses were run for 300 million generations (sampling every 30,000 steps). MCMC convergence was confirmed by examining the likelihood plots using TRACER 1.6.0 (Drummond & Rambaut 2007).

In addition to these single-locus approaches, a multilocus approach was implemented by creating a concatenated data set of all three loci. This concatenated data set was run once as an unpartitioned sequence in BEAST, using the same procedure as for the single locus data. The concatenated data were analyzed in a partitioned analysis using RAxML-HPC BlackBox (Stamatakis 2014), a phylogenetic tree inference program using maximum likelihood and rapid bootstrapping on XSEDE, through the CIPRES supercomputer portal.

For all maximum likelihood phylogenetic trees, bootstrap values of 95 and above were considered strong support, following Felsenstein (1986), though Hillis & Bull (1993) maintain that bootstrap values of 70 or above may be considered strong support. For all Bayesian phylogenetic trees, posterior values of 0.95 and above were considered strong support, following Wilcox et al. (2002) and Alfaro et al. (2002).

TCS v.1.21 was utilized to visualize sample haplotype relationships using statistical parsimony networks. The default settings were used to create the most parsimonious branch connections at the 95% confidence level (Clement et al. 2000). Between-haplotype pairwise distance (p-dist) was calculated in MEGA 5.2.2.

Hierarchical Bayesian species trees for the Bermuda, Florida Clade 1, and Florida

Clade 2 populations were estimated using *BEAST v. 1.5.3 (Heled & Drummond 2010). All three loci were used in *BEAST analyses. MCMC analyses were run for 200 million generations (sampling every 20,000 steps). Again, MCMC convergence was confirmed by examining the likelihood plots using TRACER 1.6.0 (Drummond & Rambaut 2007).

Bayesian species delimitation was conducted using Bayesian Phylogenetics and Phylogeography (BPP v.2.0 Yang & Rannala 2015). The BPP analysis is a Bayesian Markov chain Monte Carlo (MCMC) program for analyzing DNA sequence alignments using the multispecies coalescent model (Rannala & Yang 2003; see also Takahata et al. 1995). The BPP program accounts for the coalescent process in both the modern and ancestral species and the resultant gene tree-species tree conflicts, allowing a phylogeny to be estimated even if the loci display weak information (Heled & Drummond 2010). An unguided (1,1) BPP run was performed once, emulating Leaché & Fujita (2010)'s use of this chimeric approach, which utilizes both discovery and validation methods to assess speciation posterior values. Additionally, two (1,0) BPP runs were performed using two distinct guide trees (see Fig. 14 & Fig. 15). Speciation posterior values greater than or equal to 0.95 were considered strong support (Leaché & Fujita 2010; Satler et al. 2013).

Results

The Bayesian and maximum likelihood phylogenetic trees for COI displayed consistently strong bootstrap (≥96) and posterior values (≥ 0.99) at nodes delimiting the populations into three reciprocally monophyletic clades. However, both trees exhibited low support for the resolution of the relationship among these clades (see Fig. 3 & Fig. 4). The Bayesian phylogenetic tree extrapolated from the 16S data set provides a similar evaluation, with strong posterior support (≥0.99) of three distinct clades, but poor

resolution of the exact relationships between them (see Fig. 5). The maximum likelihood phylogenetic tree using the 16S data set displays strong support (bootstrap values ≥96) for the nodes delimiting the two Florida clades, but weak support for the node delimiting the Bermuda clade (see Fig. 6).

The Bayesian and maximum likelihood phylogenetic trees constructed using the enolase locus are less definitive than the trees from the mitochondrial data. In the enolase trees, there is widespread mixing of samples collected from Bermuda, Florida Clade 1, and Florida Clade 2, and exceedingly poorly supported nodes (see Fig. 7 & Fig. 8). The Bayesian phylogenetic tree constructed using the unpartitioned concatenated data provides a strongly supported delimitation into three clades (posterior values of ≥0.99 at each node). As with the COI trees, however, the resolution of the relationship between these three clades is not fully resolved (see Fig. 9). The maximum likelihood phylogenetic tree using partitioned concatenated data exhibited strong bootstrap support (100) at the two nodes delimiting the Florida clades, but did not resolve the Bermuda samples into a distinct clade (probably due to the lack of an outgroup) (see Fig. 10).

The hierarchical Bayesian analysis using partitioned concatenated multilocus data delimited the Florida Clade 1, Florida Clade 2, and Bermuda populations as three separate species (see Fig. 11). In this tree, the Bermuda clade and Florida Clade 2 were designated as closest sister taxa, but the relationship displayed a weak posterior value (0.6802).

Both guided (1,0) and unguided (1,1) BPP species delimitation programs also supported the 3-species model (see Fig. 13). Posterior values of 1.0 for all three species were produced by both the guided and unguided delimitations, which surpass the 0.95 typically required of speciation events (Leaché & Fujita 2010; Satler et al. 2013). In

constructing a species tree, however, the best tree produced by the unguided program only returned the weak posterior value of 0.75863. Both guided runs returned posterior values of 1.0 for their respective guide trees, thus producing an inconclusive result. So, although the BPP program recognized three well-defined species, the nature of the species tree remains unresolved.

Pairwise distances calculated between the populations using the COI locus indicate that the populations differ genetically 5.7% to 7.4% (Table 1). This divergence exceeds the 3% level of difference commonly reported for this marker in distinct but related species (Plaisance et al. 2009). The pairwise distances calculated between the populations for 16S locus were lower, 2.5% to 3.2% (see Table 1). These lower values are consistent with the lower variability and slower evolution of 0.4 to 0.9% per million years for 16S (Bilodeau et al. 2005) compared to COI. The nuclear gene for enolase had much lower pairwise distances (<0.5%), unexpected groupings in the phylogenetic analysis, and little support for those groupings.

Statistical parsimony (TCS) analysis using the COI data set produced three discreet haplotype networks (see Fig. 12); a large haplotype network comprising the 36 Bermuda samples, and two smaller discreet haplotype networks within the Florida population (described in Titus & Daly 2015).

Discussion

These findings using molecular multilocus phylogenetic analyses support the designation of the Bermuda population of Pederson's cleaner shrimp as a new species, *Ancylomenes anthophilus*. These results support the finding by Titus and Daly (2015) of cryptic species in the Florida Keys. The discrepancy in pairwise distance and tree support values between the nuclear and mitochondrial genes may reveal that these lineages recently diverged in the last 1-2 million years, as diploid nuclear genes require significantly longer to become distinctly sorted compared to their haploid mitochondrial counterparts (Negel & Avise 1986; Moore 1995).

The inability to establish a well-supported tree for these species using the afore-mentioned phylogenetic analyses indicates an unresolved polytomy. A possible means to resolve this uncertainty could be increased sampling of the populations; Zwickl & Hillis (2002) and Heat et al. (2008) found that augmenting taxon sampling improves phylogenetic accuracy. This study used a randomly sampled subset of the total available sequenced samples for the three clades, so analyzing the remainder of the samples could aid in producing a more robust phylogeny. Increasing taxon sampling to include samples collected from other sites across the Caribbean could also articulate other relationships between the populations. Sampling additional loci could further aid in resolving the phylogeny, especially if one or more of the loci already sampled were not completely sorted (Maddison & Knowles 2006; McCormack et al. 2009). Finally, the inclusion of an outgroup species (such as *Periclimenes yucatanicus,* another Caribbean caridean shrimp) could enhance resolution of the maximum likelihood phylogenetic trees (Hayes et al. 2009; Smith 2008). The inclusion of an outgroup would probably not enhance resolution for the

Bayesian phylogenetic trees produced in BEAST (Drummond & Rambaut 2007; Suchard & Rambaut 2009).

Furthermore, a fertile future source of research lies in examining whether the Bermuda population has undergone an ecological shift commensurate with its divergence as a species, and thus differs in cleaning practices or host specificity. Nizinski (1989) reported some evidence that such a change in ecological role may have occurred, as 40 hours of field analysis in Bermuda revealed no cleaning activity. This apparent reduction in cleaning contrasts with studies in the Virgin Islands and the Netherlands Antilles that describe *A. pedersoni* as a major, effective cleaner (Wicksten 1995a; Huebner & Chadwick 2012; McCammon et al. 2010). If Nizinski's (1989) findings accurately represent the decreased cleaning role of *A. anthophilus* in the Bermuda reef ecosystem, perhaps its divergence from its Caribbean neighbors has been accompanied by the adoption of a more facultative rather than obligate cleaning strategy. More research will have to be conducted to test whether cleaning opportunities in the Bermuda reef system are less available than in other Caribbean locations.

The next important question for investigation in this system will be to determine how these populations evolved and diverged. Specifically, the Bermuda population might have been founded by a chance colonization event, which combined with prolonged isolation might lead to allopatric speciation. Conversely, the Bermuda and Florida clades might have diverged while exchanging some gene flow, in a manner more consistent with sympatric speciation. These hypotheses should be evaluated using demographic tests such as Bayesian skyline plots (Drummond et al. 2005) and IMa2 (Hey 2011), which calculate possible past population dynamics.

The designation of *A. anthophilus* as an endemic Bermudan species also will provide crucial information for conservation efforts. Sterrer (1998) observed that anthropogenic impacts on the island have resulted in many extinctions of its endemic fauna, from seagulls to starfish. Furthermore, in light of the historical spate of calamitous ecological events that have befallen Bermuda, such as coral bleaching (Cook et al. 1990) and mangrove retreat (Ellison 1993), and in combination with recent threats such as climate change and ocean acidification (Hoegh-Guldberg et al. 2007), the preservation of endemic species has never been more of a concern. The continued discovery of Bermuda's cryptic diversity must remain an ongoing research goal, in order to gain a more complete measure of the array of species on the island, and to allow conservation experts and government authorities to adequately prioritize their protection.

Acknowledgments

I would like to thank my advisors, Dr. Marymegan Daly and Benjamin Titus, for their development and support of this project, and for their incredible mentorship and encouragement.

Literature Cited

Alfaro M.E., Zoller S., Lutzoni F. (2002) Bayes or bootstrap? A simulation study comparing the performance of Bayesian Markov chain Monte Carlo sampling and bootstrapping in assessing phylogenetic confidence. *Mol. Biol. Evol.*, **20**, 255–266.

Bauer R.T. (2004) Remarkable shrimps: adaptations and natural history of the carideans. University of Oklahoma Press, Norman, 316.

Bilodeau A.L., Felder D.L., Neigel J.E. (2005) Population structure at two geographic scales in the burrowing crustacean *Callichirus islagrande* (Decopoda, Thalassinidea): historical and contemporary barriers to planktonic dispersal. *Evolution*, **59**, 2125–2138.

Bowman T.E., Iliffe T.M. (1985) *Journal of Crustacean Biology*, **5**, 58–73.

Boxshall G.A., Iliffe T.M. (1990) Three new species of misophrioid copepods from oceanic islands, *Journal of Natural History*, **24**, 595–613.

Briggs J.C. (1966) Oceanic islands, endemism, and marine paleotemperatures, *Syst. Biol.,* **15**, 153–163.

Briones-Fourzan P., Perez-Ortiz M., Negrete-Soto F., Barradas-Ortiz C., Lozano-Alvarez E. (2012) Ecological traits of Caribbean sea anemones and symbiotic crustaceans. *Mar. Eco. Prog. Ser.*, **470**, 55–68.

Carstens B.C., Pelletier T.A., Reid N.M., Satler J.D. (2013) How to fail at species delimitation. *Mol. Eco.*, **22**, 4369–4383.

Chace F.A. (1958) A new genus of shrimp from the genus *Periclimenes* found in the West Indies. *Proc. Biol. Soc. Washington*, **71**, 125–132.

Clement M., Posada D., Crandall K.A. (2000) TCS: a computer program to estimate gene genealogies. *Mol. Eco.*, **9**, 1657–1659.

Cook C.B., Logan A., Ward J., Luckhurst B., Berg C.J. (1990) Elevated temperatures and bleaching on a high-latitude reef: the 1988 Bermuda event. *Coral Reefs*, **9**, 45–49.

DeGrave S., Li C.P., Tsang L.M., Chu K.H., Chan T. (2014) Unweaving hippolytoid systematics (Crustacea, Decapoda, Hippolytidae): resurrection of several families. *Zoological Scripta*, **43**, 496–507.

Drummond A.J., Rambaut A., Shapiro B., Pybus O. G. (2005) Bayesian coalescent inference of past population dynamics from molecular sequences. *Mol. Biol. Evol.*, **22**, 1185–1192.

Drummond A.J., Rambaut A. (2007) "BEAST: Bayesian evolutionary analysis by sampling trees." *BMC Evolutionary Biology*, **7**, 214.

Edgar R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nuc. Acids Res.*, **32**, 1792–1795.

Ellison J. C. (1993) Mangrove retreat with rising sea level, Bermuda, *Estuarine, Coastal, and Shelf Science*, **37**, 75–87.

Felsenstein J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39,** 783–791.

González S., Maldonado J.E., Leonard J.A., Vila C., Barbanti Duarte J.M., Merino M., Brum-Zorilla M., Wayne R.K. (1998) Conservation genetics of the endangered pampas deer (*Ozotoceros besoarcticus*). *Mol. Eco.*, **7**, 47–56.

Hare J.A., Churchill J.H., Cowen R.K., Berger T.J., Cornillon P.C., Dragos P., Glenn S.M., Govoni J.J., Lee T.N. (2002) Routes and rates of larval fish transport from the southeast to the northeast United States continental shelf. *Limnology and Oceanography*, **47**, 1774–1789.

Hayes K.A., Cowie R.H., Thiengo S.C. (2009) A global phylogeny of apple snails: Gondwanan origin, generic relationships, and the influence of outgroup choice (Caenogastropoda: Ampullariidae). *Biological Journal of the Linnean Society*, **98**, 61–76.

Held C. (2003) Molecular evidence for cryptic speciation within the widespread Antarctic crustacean *Ceratoserolis trilobitoides* (Crustacea, Isopoda), in Antarctic biology in a global context. (Huiskes A.H. ed.). Backhuys Publishers, 2003: 135–139.

Heled J., Drummond A.J. (2010) Bayesian inference of species trees from multilocus data. *Mol. Biol. Evol.*, **27**, 570–580.

Hey J. (2006) On the failure of modern species concepts. *Trends in Ecology and Evolution*, **21**, 447–451.

Hey J. (2011) Documentation for IMa2. http://genfaculty.rutgers.edu/hey/software.

Hillis D.M., Bull J.J., (1993) An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Syst. Biol.*, **42**, 182–192.

Holthuis L.H., Eibl-Eibesfeld I., A new species of the genus *Periclimenes* from Bermuda (Crustacea, Decapoda, Palaemonidae). *Bermuda Biological Station for Research*, **44**, 185–190.

Hurt C., Anker A., Knowlton N. (2009) A multilocus test of simultaneous divergence across the isthmus of Panama using snapping shrimp in the genus *Alpheus*. *Evolution*, **63**, 514–530.

Iliffe T.M., Fosshagen A. (1985) A new genus of calanoid copepod from an anchialine cave in Brazil. *Bull. Plankton Soc. Japan,* **1991**, 339–345.

Iliffe T.M., Hart Jr C.W., Manning R.B. (1983) Biogeography and the caves of Bermuda. *Nature,* **302**, 141–142.

Jansson R. (2003) Global patterns in endemism explained by past climatic change. *Proc. Roy. Acad. Bio. Sci.*, **15**, 10.1098/rspb.2002.2283.

Kumar, S., S. Subramanian. (2002) Mutation rates in mammalian genomes. *Proc. Natl. Acad. Sci. USA*, **99**, 803–808.

Larson A. (1998) The comparison of morphological and molecular data in phylogenetic systematics. In Molecular approaches to ecology and evolution. (DeSalle R., Schierwater B., eds.). Birkhauser Verlag Basel, 1998, 275–296.

Leaché A.D., Fujita M.K., (2010) Bayesian species delimitation in West African forest geckoes (*Hemidactylus fasciatus*), *Proc. Roy. Acad. Bio. Sci.*, **277**, 3071–3077.

Leakey R., Lewin R., (1996) The Sixth Extinction: biodiversity and its survival. Anchor Books, New York.

Lessios H.A. (2008) The Great American Schism: divergence of marine organisms after the rise of the Central American Isthmus. *Annual Review of Ecology, Evolution, and Systematics*, **39**, 63–91.

Maddison W.P., Knowles L.L. (2006) Inferring phylogeny despite incomplete lineage sorting, *Syst. Biol.*, **55**, 21–30.

Marchordom A., Mcpherson E., (2004) Rapid radiation and cryptic speciation in squat lobsters of the genus *Munida* (Crustacea, Decapoda) and related genera in the South West Pacific: molecular and morphological evidence. *Molecular Phylogenetics and Evolution*, **33**, 259–279.

Mayden, R.L. (1997) A hierarchy of species concepts: the denouement in the saga of the species problem. in Species: the units of biodiversity (Claridge M.F., Dawah H.A., Wilson M.R. eds.), Chapman and Hall, London, 381–424.

McCammon A., Sikkel P.C., Nemeth D. (2010) Effects of three Caribbean cleaner shrimps on ectoparasitic monogeneans in a semi-natural environment. *Coral Reefs*, **29**, 419–426.

McCormack J.E., Huang H., Knowles L.L. (2009) Maximum likelihood estimates of species trees: how accuracy of phylogenetic inference depends upon the divergence history and sampling design. *Syst. Biol.*, **58**, 501-508.

Moore W. S. (1995) Inferring phylogenies from mtDNA variation: mitochondrial-gene trees versus nuclear-gene trees. *Evolution*, **49**, 718–726.

Moriyama E.N., Gojobori T. (1992) Rates of synonymous substitution and base composition of nuclear genes in *Drosophila*. *Genetics*, **130**, 855–864.

Nizinski M.S. (1989) Ecological distribution, demography and behavioral observations on *Periclimenes anthophilus,* an atypical symbiotic cleaner shrimp, *Bull. Mar. Sci.*, **45**, 174–188.

Neigel J.E., Avise J.C. (1986) Phylogenetic relationships of mitochondrial DNA under various demographic models of speciation. *Evolutionary Processes and Theory*, 515–534.

Okuno J., Bruce A.J. (2010) Designation of *Ancylomenes* gen. nov., for the '*Periclimenes aesopius* species group' (Crustacea: Decapoda: Palaemonidae), with the description of a new species and a checklist of congeneric species. *Zootaxa*, **2372**, 85–105.

Pante E., Schoelinck C., Puillandre N. (2015) From integrative taxonomy to species description: one step beyond. *Syst. Biol.*, **64**, 152-160.

Perez-Losada M., Jara C.J., Bond-Buckup G., Crandal K.A. (2002) Conservation phylogenetics of Chilean freshwater crabs *Aegla* (Anomura, Aeglidae): assigning priorities for aquatic habitat protection. *Biological Conservation*, ***105***, 345–353.

Pimm S.L., T.M. Brooks. (2000) The Sixth Extinction: how large, where, and when? in Nature and human society: the quest for a sustainable world (Raven P.H ed.). National Academic Press, Washington DC, 46–62.

Plaisance L., Knowlton N., Paulay G., Meyer C. (2009) Reef-associated crustacean fauna: biodiversity estimates using semi-quantitative sampling and DNA barcoding. *Coral Reefs*, **28**, 977–986.

de Queiroz K. (1998) The general lineage concept of species, species criteria, and the process of speciation. in Endless forms: species and speciation. (Howard D.J., Berlocher S.H eds.) Oxford University Press, USA.

de Queiroz K. (2007) Species concepts and species delimitation. *Soc. Sys. Biol.*, **56**, 879–886.

Santos S.R. (2006) Patterns of genetic connectivity among anchialine habitats: a case study of the endemic Hawaiian shrimp *Halocaridina rubra* on the island of Hawaii. *Mol. Eco.*, **15**, 2699–2718.

Satler J.D., Carstens B.C., Hedin M. (2013) Multilocus species delimitation in a complex of morphologically conserved trapdoor spiders (Mygalomorphae, Antrodiaetidae, *Aliatypus*). *Syst. Biol.*, **62**, 805–823.

Schultz E.T., Cowen R.K. (1994) Recruitment of coral-reef fishes to Bermuda – local retention or long-distance transport. *Mar. Eco. Prog. Ser.*, **109**, 15–28.

Shaffer B.H., Meylan, P., McKnight M.L. (1997) Tests of turtle phylogeny: molecular, morphological and paleontological approaches. *Syst. Biol.*, **46**, 235–268.

Silbiger N. J., Childress M. J., (2008) Interspecific variation in anemone shrimp distribution and host selection in the Florida Keys (USA): implications for marine conservation. *Bull. Mar. Sci.*, **83**, 329–345.

Smith A.B. (2008) Rooting molecular trees: problems and strategies. *Biological Journal of the Linnaean Society*, **51**, 279-292.

Spotte S. (1999) Possible synonymy of the western Atlantic anemone shrimps *Periclemines pedersoni* and *P. anthophilus* based on morphology. *Bull. Mar. Sci.*, **65**, 407–417.

Stamatakis, A. (2014) RAxML Version 8: A tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies. Bioinformatics 10.1093/bioinformatics/btu033.

Sterrer W. (1998) Changes in Bermuda's biota, *Boletim do Museu Municipal do Funchal*, 459–453.

Sterrer W., Schoepfer-Sterrer. C. (1986) Marine fauna and flora of Bermuda. *Wiley-Science*, New York.

Suchard M.A., Rambaut A. (2009) "Many-core algorithms for statistical phylogenetics" *Bioinformatics*, **25**, 1370–1376.

Takahata N., Satta Y., Klein J., (1995) Divergence time and population size in the lineage leading to modern humans. *Theor. Popul. Biol.*, **48**, 198–221.

Tamura K., Peterson D., Peterson N., Stecher G., Nei M., Kumar S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. and Evo.*, **28**, 2731–2739.

Titus B., Daly M. (2015) Fine-scale phylogeography reveals cryptic biodiversity in Pederson's cleaner shrimp, *Ancylomenes pedersoni* (Crustacea: Caridea: Palaemonidae), along the Florida Reef Tract. *Marine Ecology*, **36**, 1379–1390.

Tolley K.A., Groeneveld J.C., Gopal K., Matthee C.A., (2005) Mitochondrial DNA panmixia in spiny lobster Palinurus gilchristi suggests a population expansion, *Mar. Ecol. Prog. Ser.*, **297**, 225–231.

Tsang, L.M., Chan, T.Y., Ahyong, S., Chu, K.H. (2011) Hermit to king, or hermit to all: multiple transitions to crab-like forms from hermit crab ancestors. *Syst. Biol.*, **60**, 616–629.

Wicksten M.K. (1995a) Associations of fishes and their cleaners on coral reefs of Bonaire, Netherlands Antilles. *Copeia*, **1995**, 477–481.

Wicksten M.K. (1995b) Within-species variation in *Periclimenes yucatanicus* (Ives), with taxonomic remarks on *P. pedersoni* Chace (Crustacea: Decapoda: Caridea: Palaemonidae). *Proc. Bio. Soc. Washington*, **108**, 458–464.

Wilcox T.P., Zwickl D.J., Heath T.A., Hillis D.M. (2002) Phylogenetic relationships of the dwarf boas and a comparison of Bayesian and bootstrap measures of phylogenetic support. *Molecular Phylogenetics and Evolution*, **25**, 361–371.

Yang Z., Rannala B. (2010) Bayesian species delimitation using multilocus sequence data. *Proc. Natl. Acad. Sci. USA*, **107**, 9264–9269.

Yang Z. (2002) Likelihood and Bayes estimation of ancestral population sizes in Hominoids using data from multiple loci. *Genetics*, **162**, 1811–1823.

Zwickl D.J., Hillis D.M. (2002) Increased taxon sampling greatly reduces phylogenetic error**.** *Syst. Biol.*, **51**, 588–598.

Tables

Table 1: Between-haplotype pairwise distances (p-dist) between Bermuda, Florida Clade 1, and Florida Clade 2.

Clades Compared	COI average p-dist	16S average p-dist	Enolase average p-dist
Bermuda & Florida Clade 1	5.7%	2.5%	0.1%
Bermuda & Florida Clade 2	5.9%	3.2%	0.1%
Florida Clade 1 & Florida Clade 2	7.5%	2.9%	0.2%

Figures

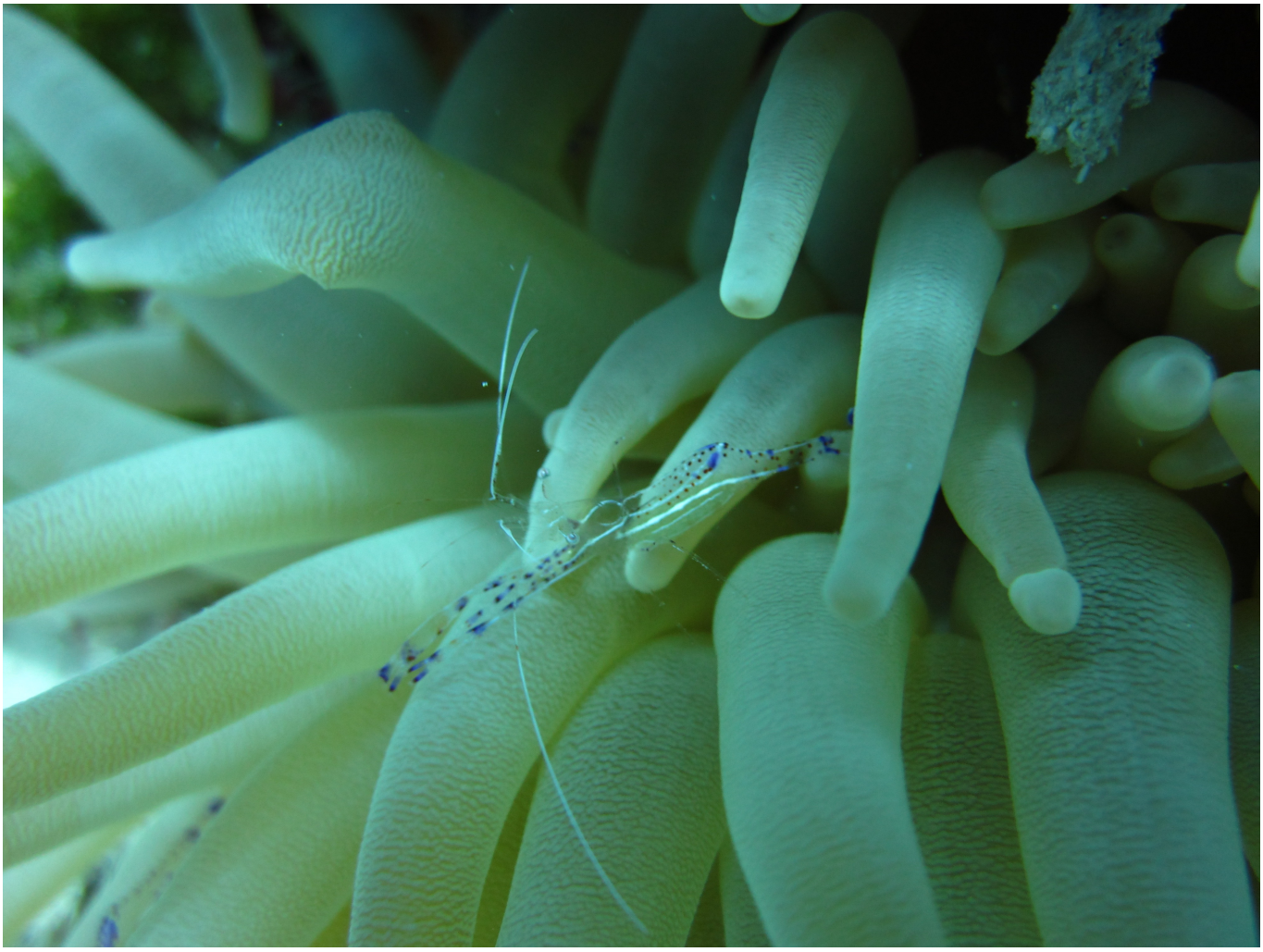


Fig. 1. Photo of Pederson's cleaner shrimp in Bermuda.

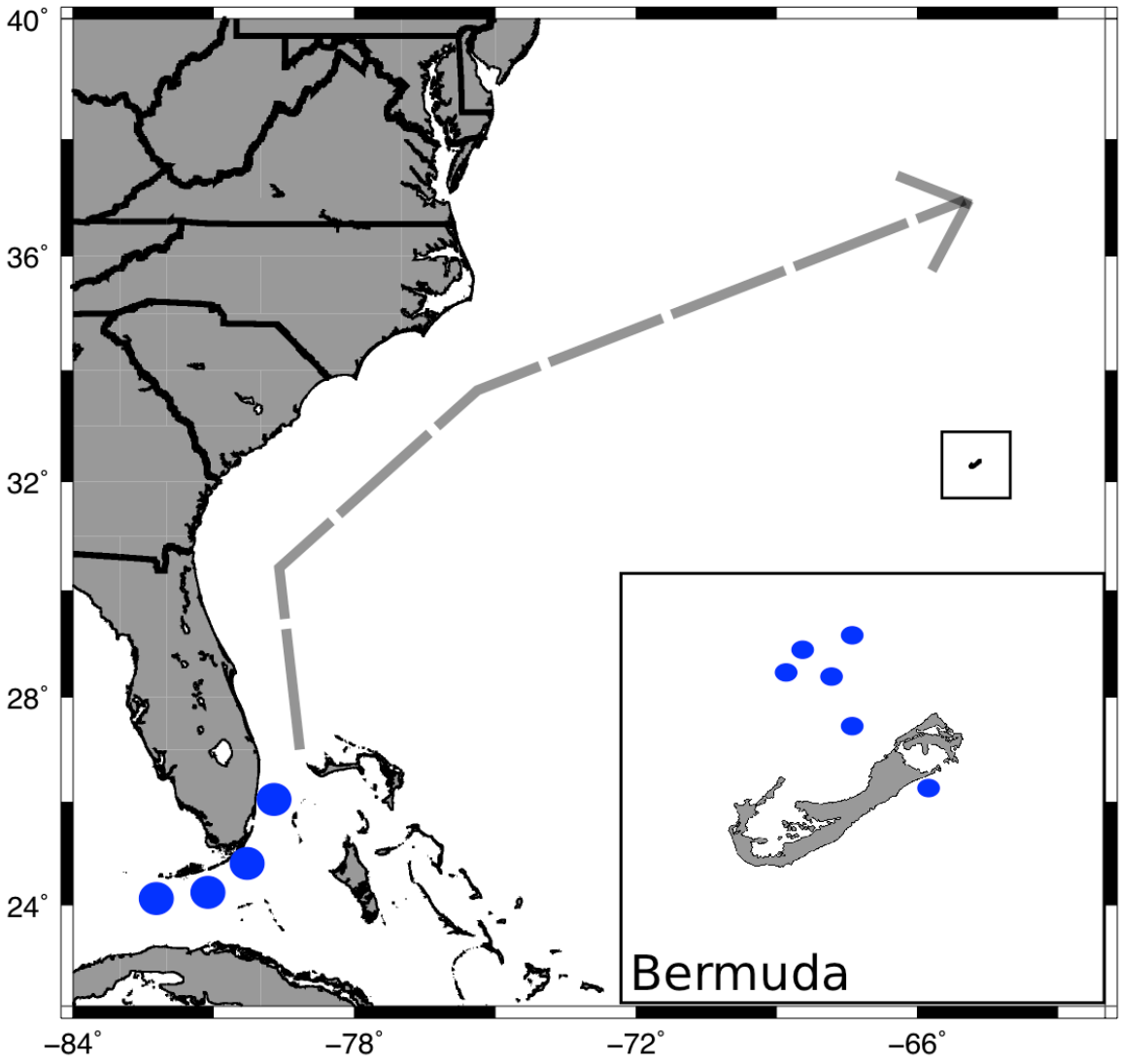


Fig. 2. Map of the Bermuda and Florida Keys. Blue dots represent major sample localities and dashed arrow represents Gulf Stream current and direction.

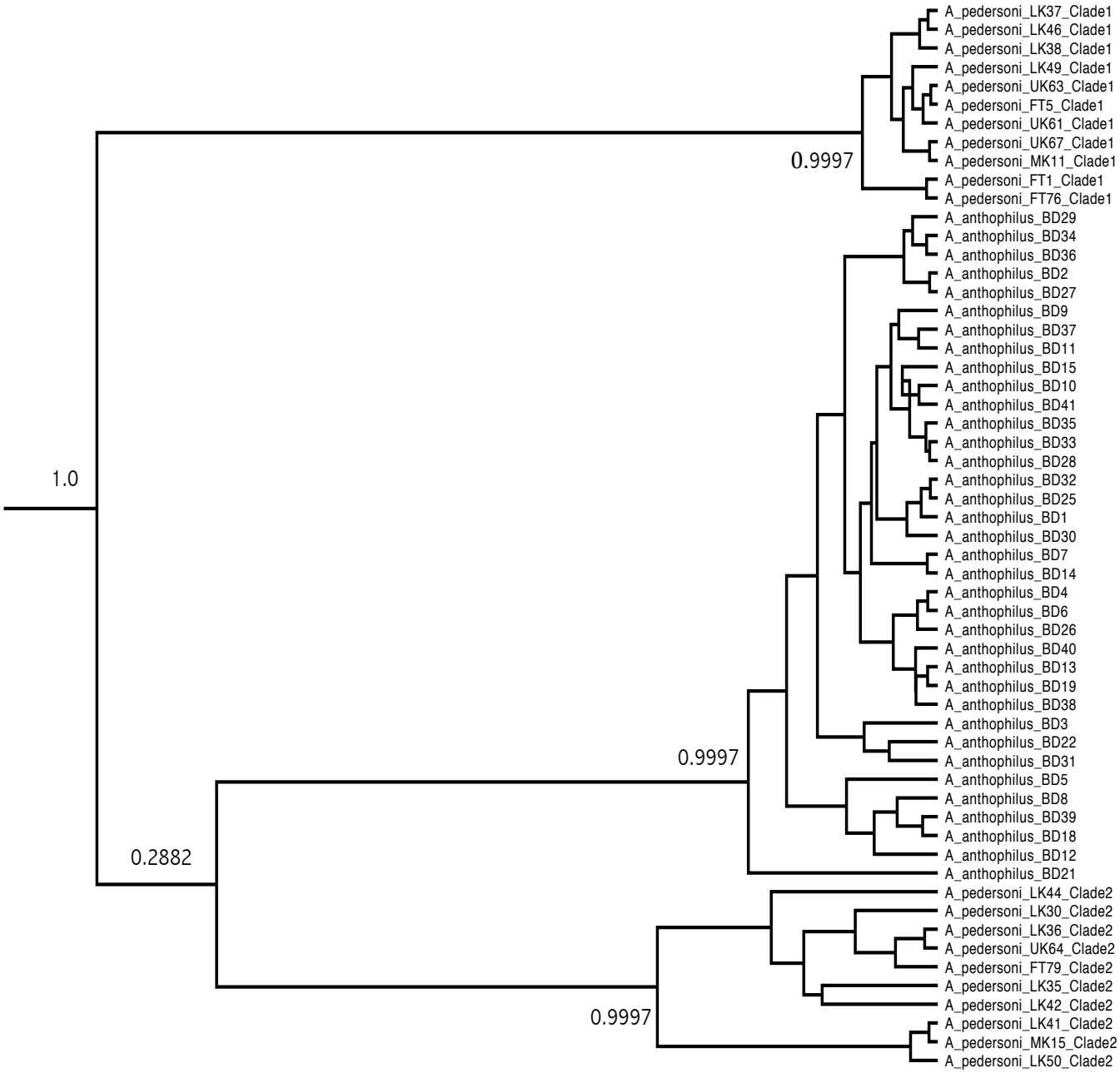


Fig. 3. Bayesian phylogenetic tree (rooted, time-measured) extrapolated in BEAST using the cytochrome c oxidase subunit I (COI) data set. Values next to tree branches represent posterior support values. See Materials and Methods for site codes.

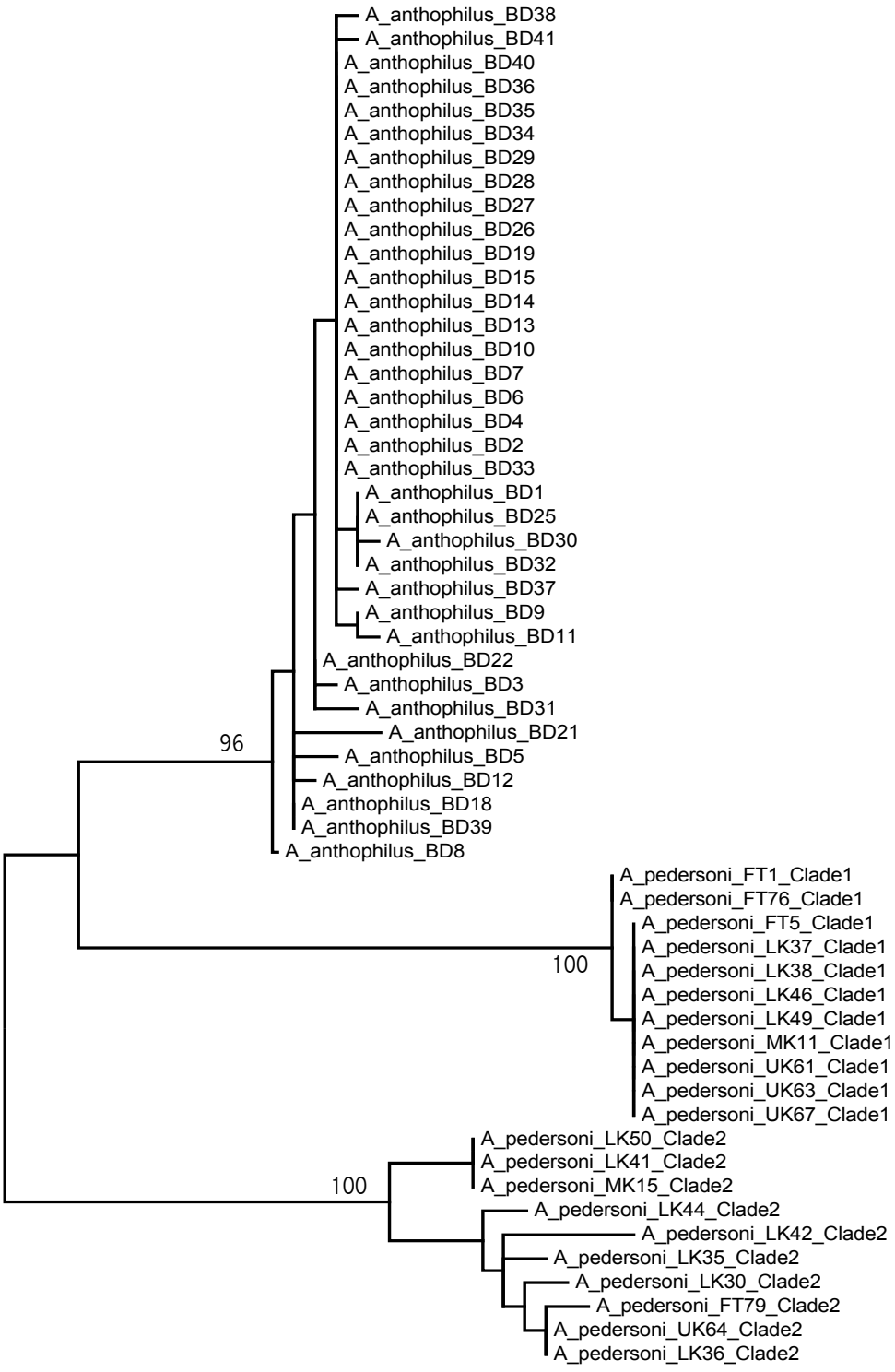


Fig. 4. Maximum likelihood phylogeny extrapolated for Ancylomenes pedersoni using the cytochrome c oxidase subunit I (COI). Values next to tree branches represent bootstrap support as percentages of 1000 re-samplings. See Materials and Methods for site codes.

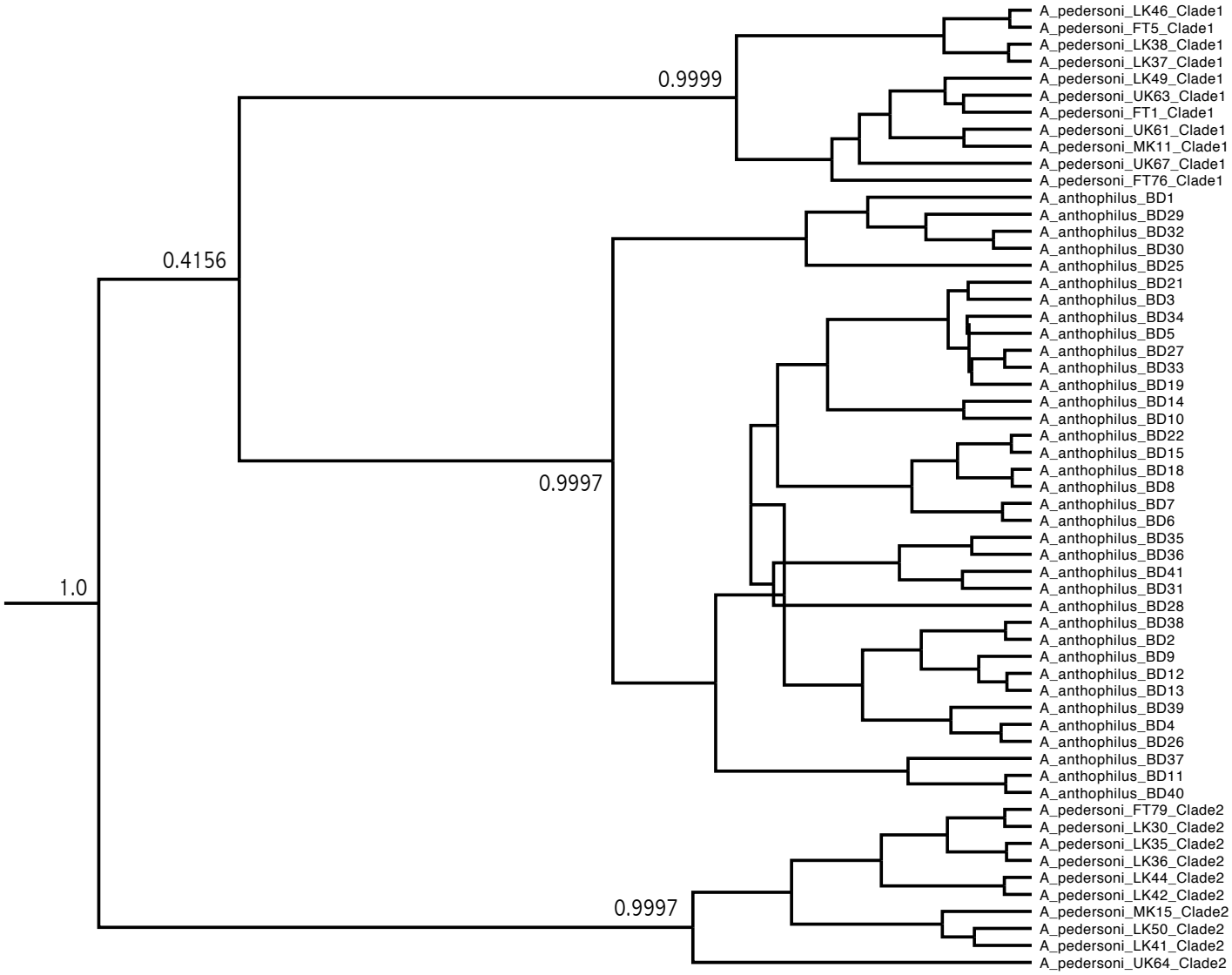


Fig. 5. Bayesian phylogenetic tree (rooted, time-measured) extrapolated using the 16S-rDNA data set data set. Values next to tree branches represent posterior support values. See Materials and Methods for site codes.

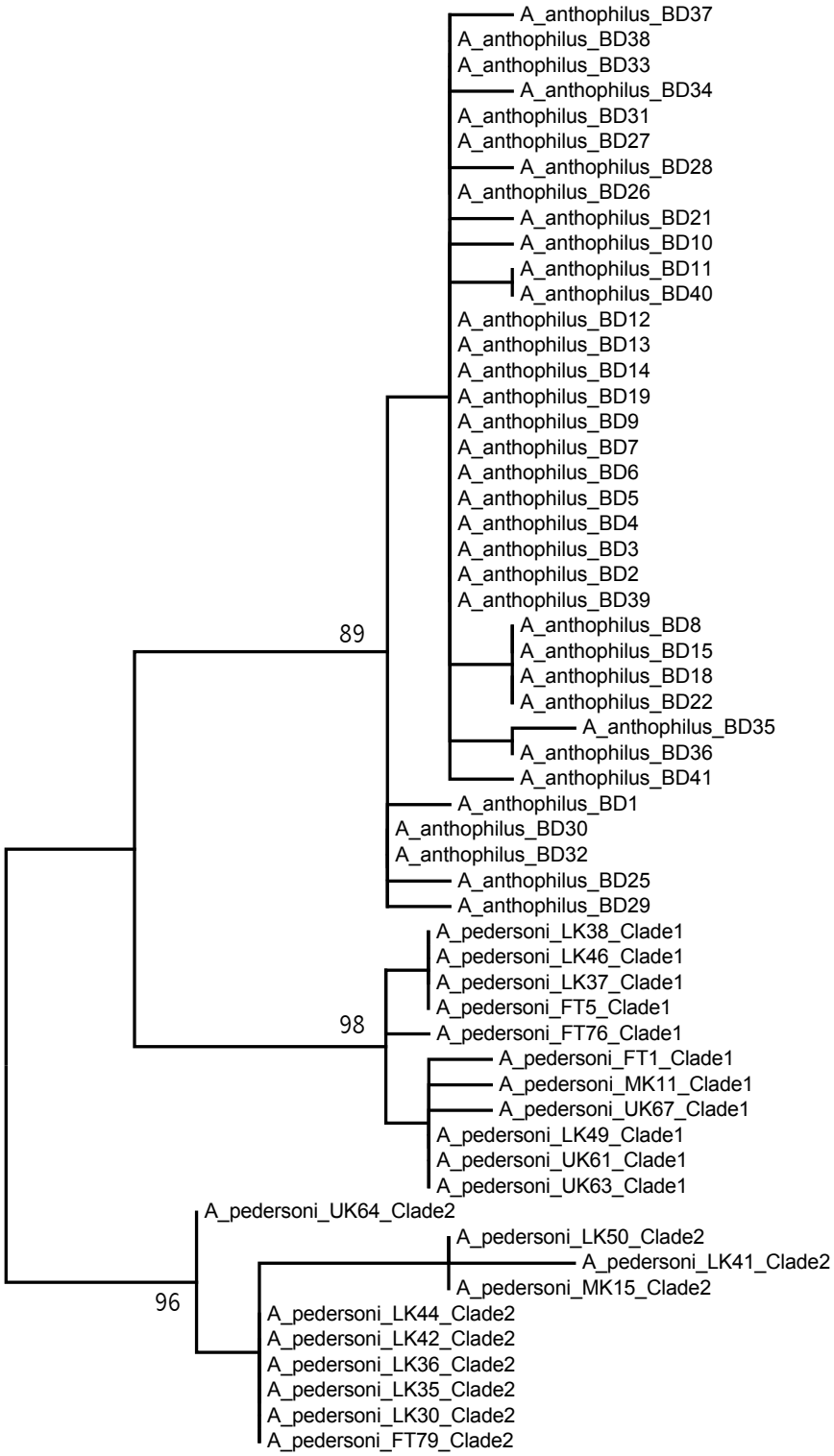


Fig. 6. Maximum likelihood phylogeny extrapolated using the 16S-rDNA data set. Values next to tree branches represent bootstrap support as percentages of 1000 re-samplings. See Materials and Methods for site codes.

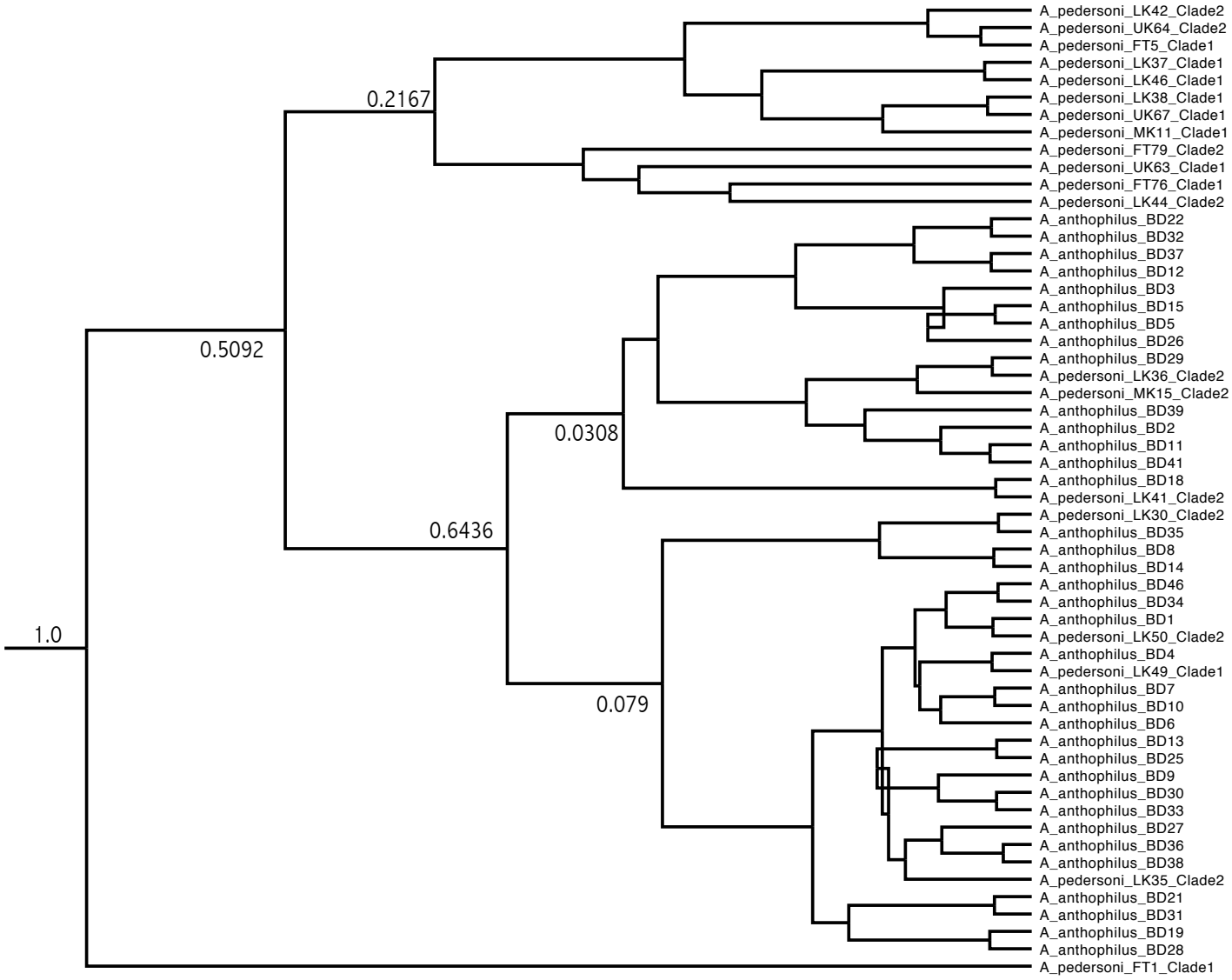


Fig. 7. Bayesian phylogenetic tree (rooted, time-measured) extrapolated using the nuclear enolase data set. Values next to tree branches represent posterior support values. See Materials and Methods for site codes.

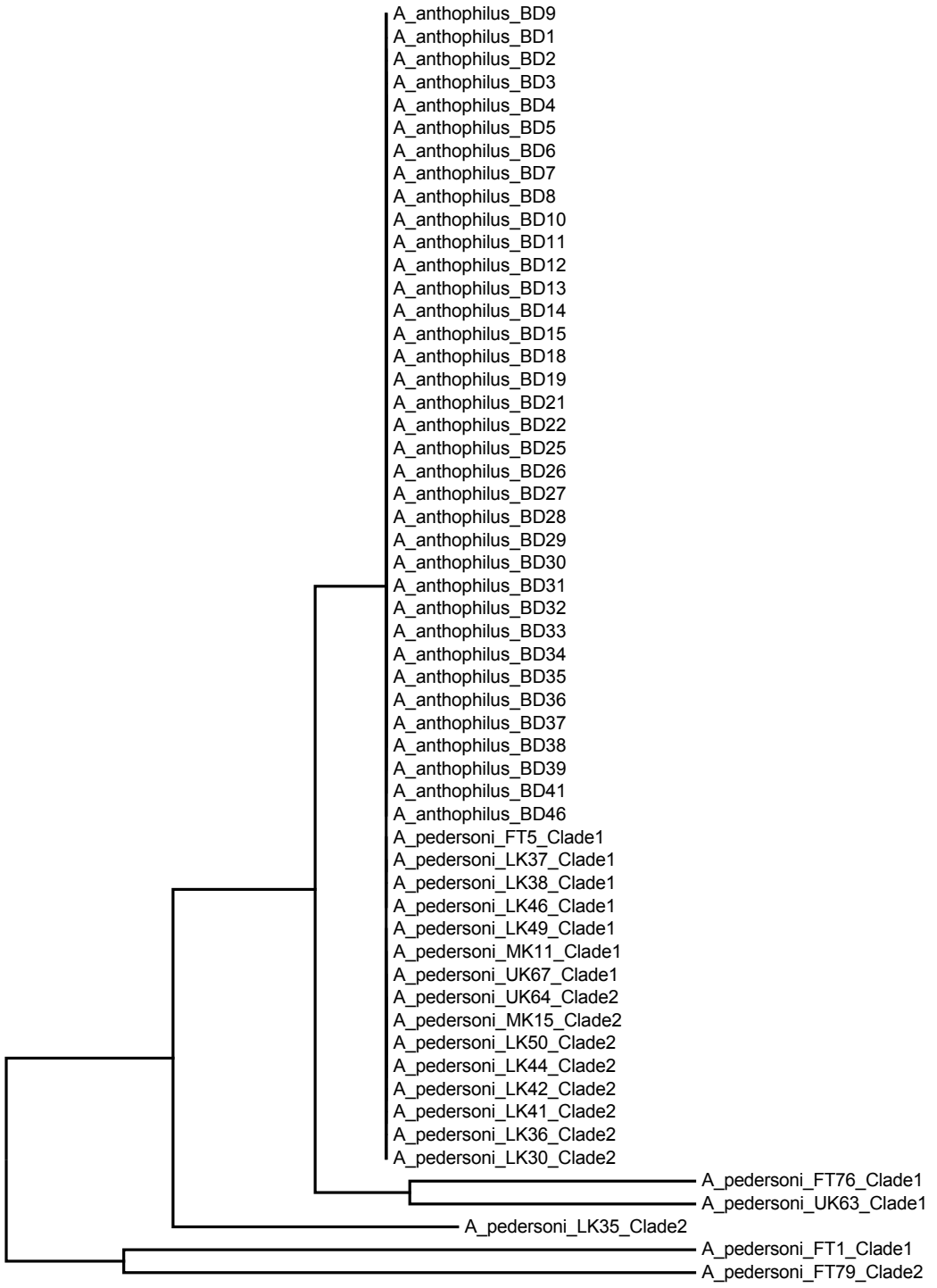


Fig. 8. Maximum likelihood phylogeny extrapolated using the nuclear enolase data set. Values next to tree branches represent bootstrap support as percentages of 1000 re-samplings. No bootstrap values above 14. See Materials and Methods for site codes.

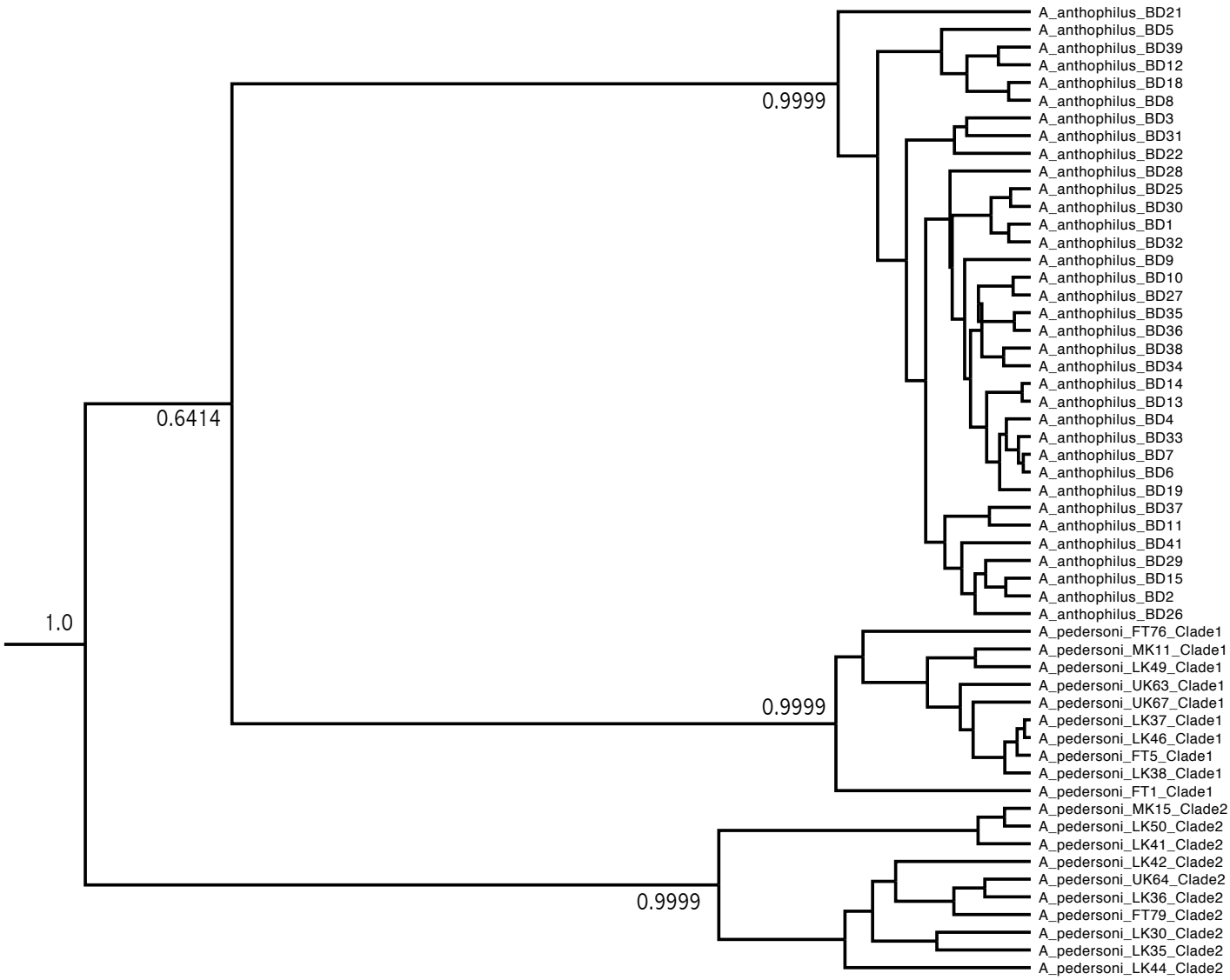


Fig. 9. Bayesian phylogenetic tree (rooted, time-measured) extrapolated using the unpartitioned concatenated multilocus data set. Values next to tree branches represent posterior support values. See Materials and Methods for site codes.

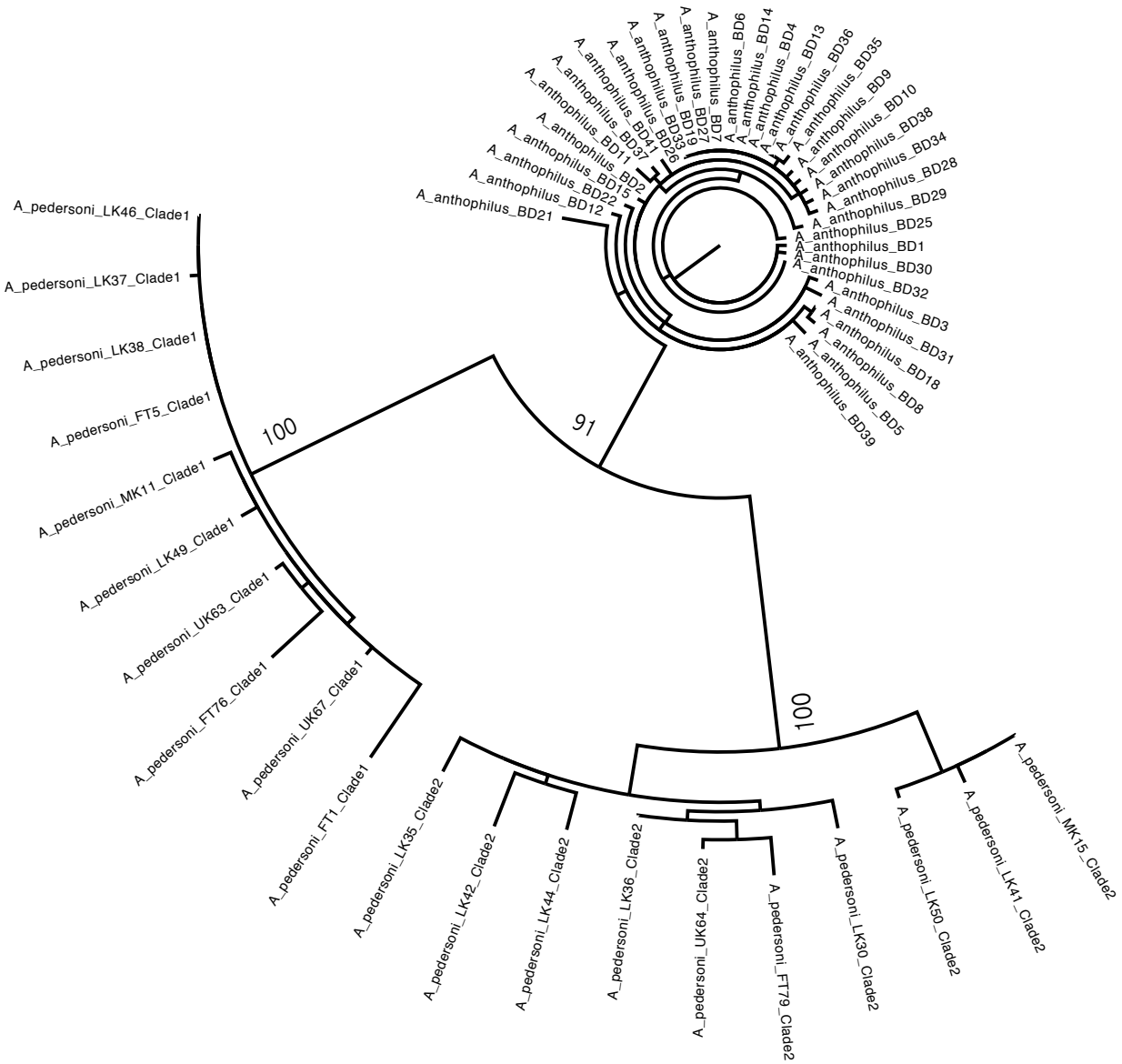


Fig. 10. Maximum likelihood phylogeny extrapolated using the unpartitioned concatenated multilocus data set. Values next to tree branches represent bootstrap support as percentages of 1000 re-samplings. See Materials and Methods for site codes.

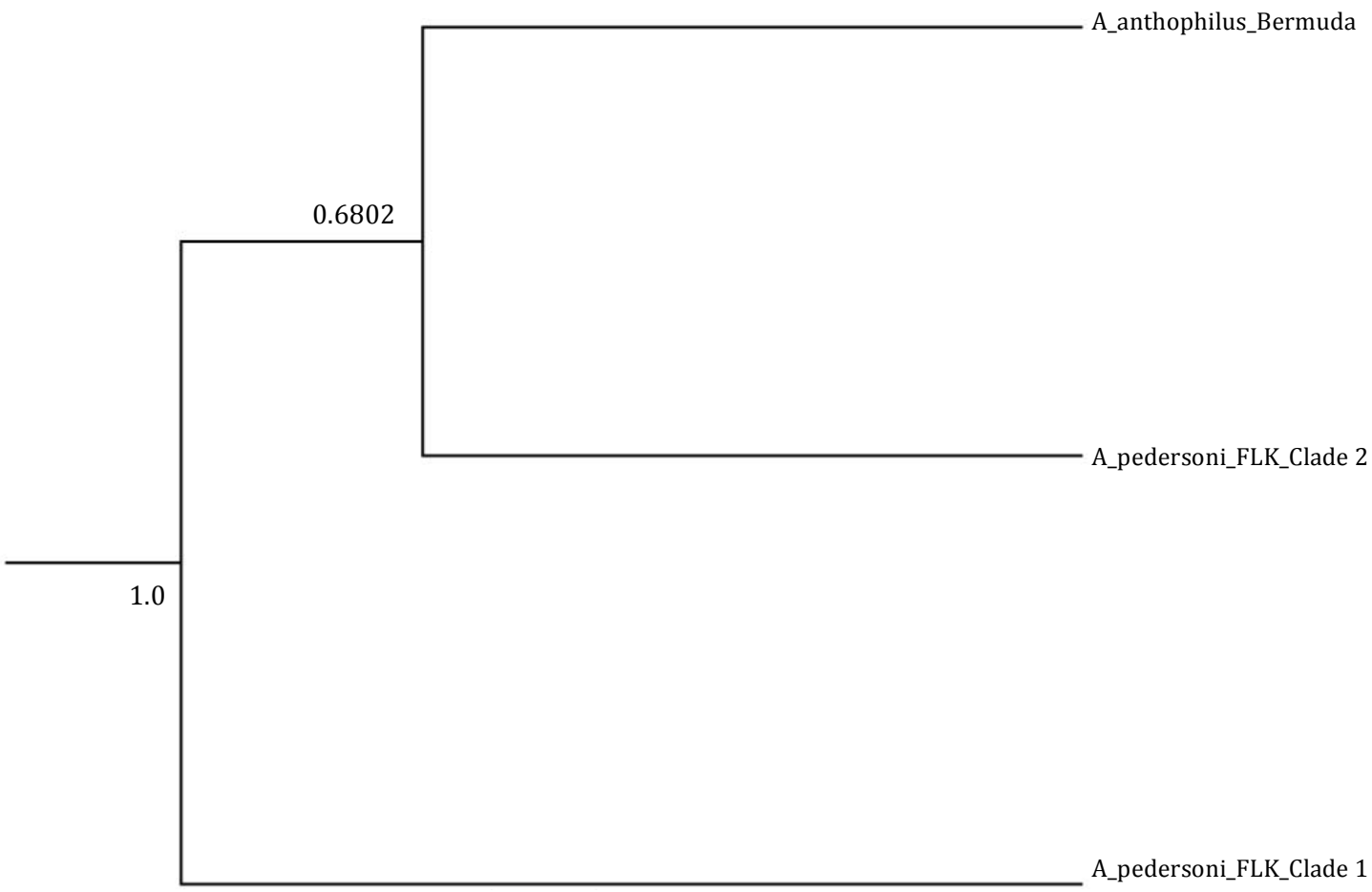


Fig. 11. Species tree estimated using *Beast, with partitioned concatenated multilocus data. Values next to tree branches represent posterior support values. See Materials and Methods for site codes.

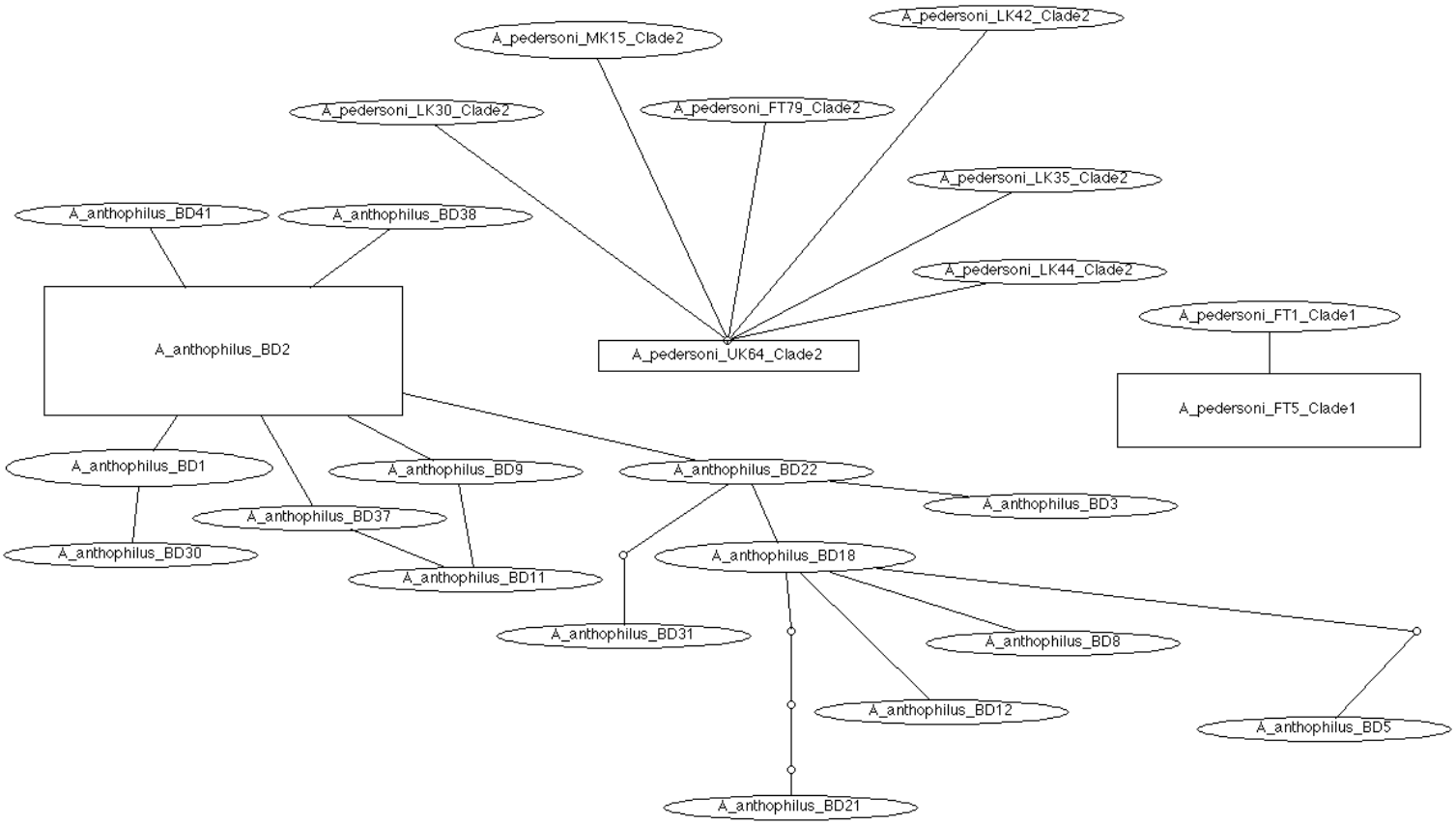


Fig. 12. Haplotype relationships for cytochrome c oxidase subunit I (COI) data set derived from TCS statistical parsimony networks.

Delimitation Method	Posterior Values		
BPP (1,0) guided	1.0	1.0	1.0
BPP (1,1) unguided	1.0	1.0	1.0
	Bermuda	**Florida Clade 2**	**Florida Clade 1**

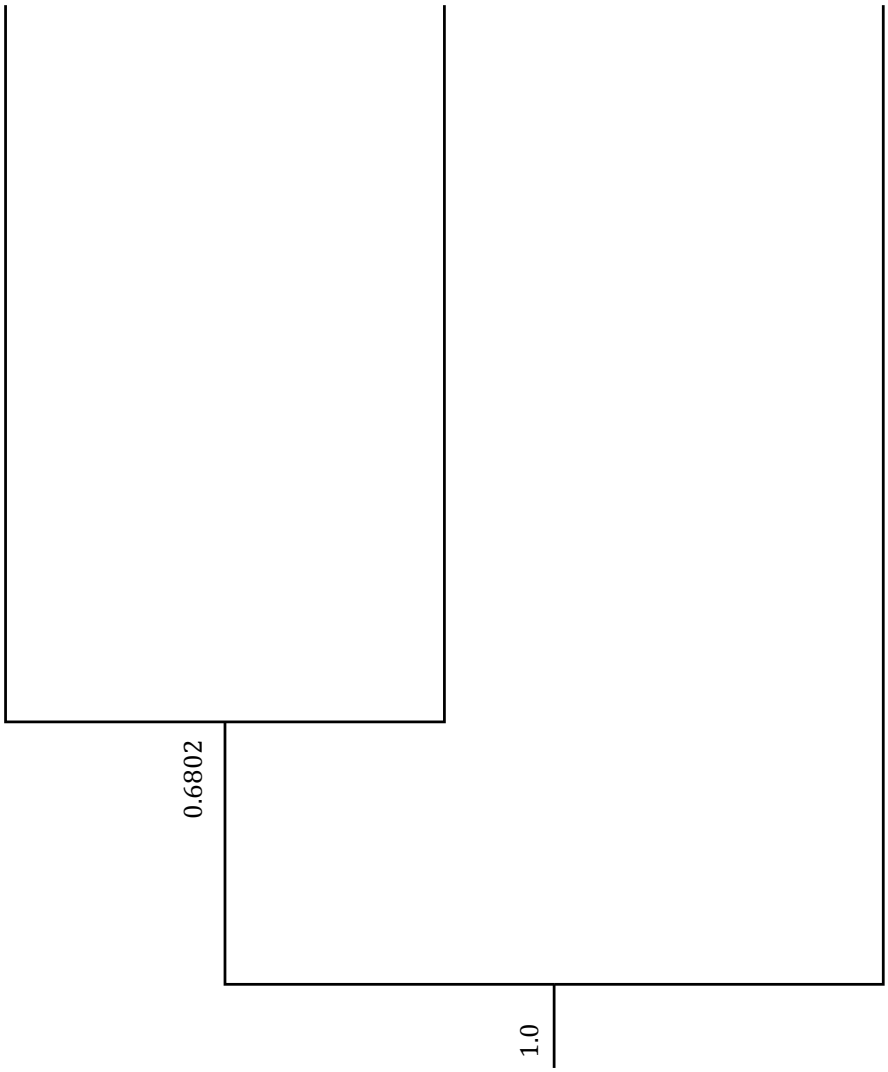
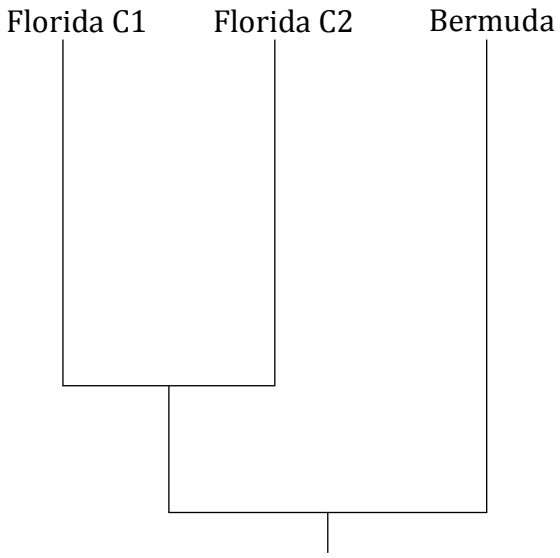


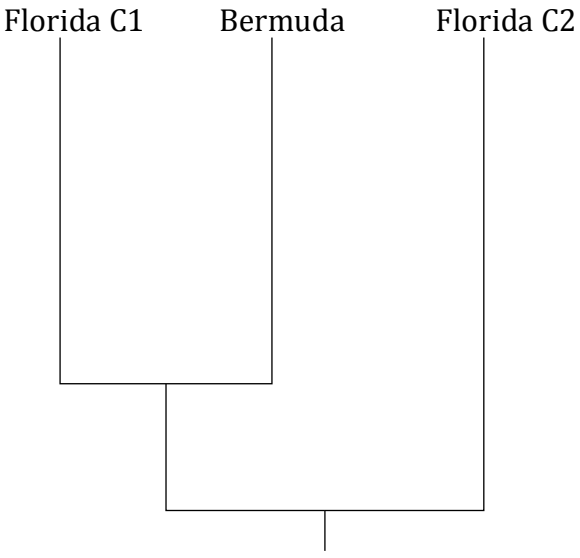
Fig. 13. Species delimitation of three clades using BPP (guided and unguided).

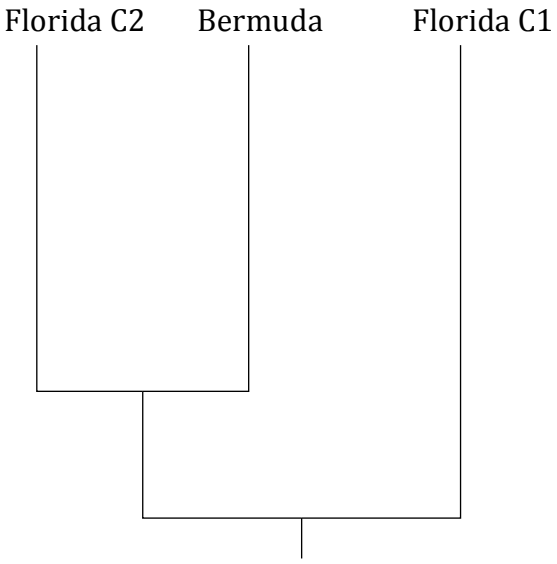
BPP (1,1) unguided: best tree



Posterior: 0.75863

BPP (1,0) guided, with two guide trees:





Posterior: 1.0

Posterior: 1.0

Fig 14. Species tree of three clades evaluated using BPP (unguided and guided).